The present invention relates to active transfer of haptens, proteins, peptides, nucleic acids and other molecules into cells. More particularly, the present invention relates to novel polypeptides which can effectively penetrate into cells, in particular eukaryotic cells, and transport thereto a substance of interest which is capable of constituting novel antiviral compositions. This invention is of major importance as it has application in a variety of fields, in particular that of gene therapy and vaccines.

Gene therapy remains dependent on a considerable number of parameters, among them the development of vectors which are capable of transferring active principles endowed with predetermined specific properties to the cytoplasm of cells of the host organism under consideration in the absence of genetic alterations associated with the use of such vectors, and with no degradation of the biological activity of the transferred active principles. Current knowledge is that in spite of the effort expended in developing vectors of viral or non viral origins, not all of these conditions have been satisfactorily fulfilled.

Further, the possibility of transporting substances efficiently into cells is also important for all biotechnological applications. Thus transferring substances into cells *in vitro* or *ex vivo* can be used either to produce proteins or peptides, or to regulate gene expression, or to analyse the properties of a given substance in that cell. *In vivo*, the transfer of a substance to a cell can also act to create models for studying diseases in animals or for studying the effect of a given compound on an organism.

The present invention thus aims to provide a novel type of vector which is both effective and is more innocuous than viral vectors in current use.

Patent application FR N°9508316 describes the use of antibodies or their F(ab')2 and Fab' fragments which can penetrate into the interior of living cells, as immunovectors for intracytoplasmic and intranuclear transfer of biologically active substances. While such vectors are highly effective, their use can produce problems in some applications. The use of antibodies or F(ab')2 antibody fragments involves the production of high titers of these molecules with qualities which are compatible with therapeutic use. Further, the use of molecules with the size and complexity of antibodies can constitute a further disadvantage as regards use. United States patent US-A-5 635 383 illustrates a further type of complex vector based on polylysine for transferring nucleic acids into cells.

The present application relates to novel polypeptides with advantageous properties both for transferring of substances into cells. The primary structure of these polypeptides is much simpler than antibodies and they are of reduced size. Further, preparation is easy and their potential applications are highly varied.

More particularly, the present invention stems from the discovery by the inventors that it is possible to identify, from whole antibodies, limited regions carrying a cellular penetration activity. The invention also stems from the discovery that it is possible to isolate, from whole antibodies, in particular from a single chain of these antibodies, peptides or polypeptides endowed with cell penetration activity. The present invention

constitutes the first demonstration that a fragment of a single chain of an antibody can effectively penetrate into cells. The present invention also constitutes the first demonstration that such a fragment is also capable, advantageously, of transporting a substance of interest into said cell, and can preferably have an antiviral activity.

The present invention thus provides novel molecules which are particularly adapted to transfer biologically active substances into eukaryotic cells, particularly mammalian cells.

In a first aspect, the invention provides a polypeptide characterized in that:

- it is constituted by a unique or repeated peptide motif; and
- it comprises an amino acid sequence constitute by one or more different antibody fragment(s); and
- it is capable of penetrating into cells.

The polypeptides of the invention comprise one or more fragment(s) of an antibody. In their simplest form, antibodies (molecules from the immunoglobulin superfamily) are constituted by four chains which are associated together (for example IgG): two heavy chains H, and two light chains L (Figure 1). These four chains are associated together post-synthesis to form a molecule with a molecular weight of about 150,000 kD. The antigenic specificity of antibodies is provided by variable domains involving a number of regions of a heavy chain and a number of regions of a light chain (Figure 1).

Polypeptides can also be constituted by sequences originating from other immunoglobulin representatives such as IgM.

Each heavy chain of an antibody is composed of about 450 amino acids, and comprises different domains termed the constant domain (C), variable domain (V) and joining domains (D and J). Particular motifs are found in the variable domains, termed CDR (Complementarity Determining Region) which can readily be localised by sequence alignment (C. Janeway and P. Travers, 1996, Immunobiology, Academic Press, "The Structure of a Typical Antibody Molecule"). For an analysis of the sequences of the variable regions, reference should also be made to the article by T. T. Wu and E. Kabat (J. Exp. Med., 1970, Vol. 132, p. 211-250).

The present application stems from the demonstration that it is possible to obtain regions which are limited in size and of simple structure with particularly advantageous properties from the antibody structure. Thus, starting from a molecule which is complex (four associated chains) and large (150000 kD), the Applicant has succeeded in constructing polypeptides with a single chain, with the capability of penetrating into cells and of transporting thereto substances of interest. The properties of the polypeptides of the invention are all the more remarkable since their sequences corresponding to those of one or more fragments of only one of the chains of an antibody and thus in order to be active, there is no need for constant regions originating from a heavy chain and a light chain. Polypeptides of the invention obtained by chemical synthesis have the same properties.

The term "polypeptide" as used in the present invention defines a molecule comprising a concatenation of amino acids, with a size in the range 3 to 100 amino acids, for example less than 60 amino acids. Still more

preferably, it is a molecule comprising a concatenation of 3 to 60 amino acids, advantageously 3 to 30. Particularly preferred polypeptides advantageously comprise more than about 10 amino acids. The polypeptide of the invention can also comprise certain structural modifications, of a chemical or enzymatic nature for example. Thus the polypeptide of the invention can comprise certain functional groups which, by chemical or enzymatic reaction, can couple with another substance. The polypeptides of the invention can also be chemically modified in order to render them more resistant to proteases or less visible to the immune system. The polypeptides of the invention can be obtained by any method which is known to the skilled person, in particular by chemical synthesis, for example using peptide synthesisers, or by fragmentation or deletion from larger polypeptides, natural or otherwise. They can also be prepared using recombinant DNA techniques, by expression of a corresponding nucleic acid in a eukaryotic or prokaryotic host cell,. Clearly, they can result from combinations of these different methods.

The term "unique peptide motif" means that, in contrast to antibodies or Fab or F(ab')2 type antibody fragments, for example, the polypeptides of the invention comprise only a single chain of amino acids. The term "repeated peptide motif" means that the polypeptides of the invention can comprise different peptide blocks assembled together, optionally chemically, to form a single chain.

The term "penetrate" or "penetrating" as used in the present invention means a polypeptide which is capable of passing from the external medium to the intracellular medium, in particular into the cell cytoplasm. This

capacity can be determined in different manners, in particular using a cell penetration test comprising initial incubation of the polypeptide to be studied in the presence of culture cells followed, after fixing and permeabilisation of these cells, by revealing the presence of said polypeptide inside said cell. Revealing can be achieved by a further incubation with labelled antibodies directed against said polypeptide and detection, in the cytoplasm or in the immediate proximity of the nucleus or even in the nucleus, of the antigen-antibody type immunological reaction between the polypeptide and the labelled antibody. A previously labelled polypeptide followed by detection of said labelling in these cellular compartments can also be used for revealing. Such a cell penetration test has been described, for example, in International patent application WO 97/02840.

As indicated above, the present invention stems from demonstrating the existence of reduced regions of an antibody endowed with cell penetration properties and which can also act to transport substances of interest. More particularly, the inventors have sought the presence of regions endowed with cell penetration properties and which could be used as a vector in place of whole antibodies in the structure of certain penetrating antibodies such as those described in WO 97/02840,. To this end, the inventors have first determined the complete sequence of heavy and light chains of three particular monoclonal antibodies, J20.8, F4.1 and F14.6. These antibodies are anti-DNA antibodies, polyreactive, which are produced by hybridoma deposited at the CNCM [National Collection of Micro-organism Cultures] under numbers I-1605, I-6506 and I-1607 (see patent application

cited above). Alignment of these sequences and their comparative analysis have revealed the following remarkable elements:

- the existence of a region of very high homology (65-70%) in the CDR2 region of these three antibodies; and
- the presence, in these three antibodies, of CDR3 regions which are rich in lysine and arginine (basic amino acids).

With regard to these results, and given that the majority of peptides capable of transport and nuclear localisation are rich in lysine and arginine, the Applicants then synthesised series of polypeptides corresponding to different regions of these antibodies, and in particular to the CDR2 and CDR3 regions, and hybrid constructions in which certain of these regions were fused together (in particular a CDR2-3 peptide carrying CDR2 and CDR3 regions in succession). A biotin region was also introduced to the N-terminal side of these polypeptides, to enable them to be detected easily.

These polypeptides were then tested for their capacity to penetrate into cells. The results obtained show that, remarkably, certain of these polypeptides have the capacity to penetrate effectively into cells. In particular, the results obtained show that the group of polypeptides which comprise all or a portion of the CDR3 region are capable of penetrating into cells.

More preferably, the polypeptides of the invention are thus constituted by a unique chain comprising at least one fragment of the heavy chain of an antibody. Still more preferably, they comprise at least a fragment of the variable region of the heavy chain of an antibody.

In a particular implementation, the invention concerns polypeptides as defined above comprising all or a portion of the CDR3 region of an antibody.

Further, the results obtained have also shown that polypeptides also containing all or a portion of the CDR2 region also have the capability of penetrating into cells. To this end, polypeptides which combine all or a portion of the CDR3 region and all or a portion of the CDR2 region have entirely remarkable cell penetration capacities.

Thus in a further implementation, the polypeptides of the invention comprise all or a portion of the CDR2 region of an antibody.

In a particularly interesting implementation, the polypeptides of the invention more preferably comprise all or a portion of the CDR3 region and all or a portion of the CDR2 region. This type of polypeptide is particularly advantageous as it is capable of mass penetration into the interior of living cells.

More particularly, the expression "all or a portion" as used in the present application means that the polypeptides of the invention can comprise either the whole of the CDR region concerned of an antibody, or only a portion thereof, it being understood that the polypeptide retains a cell penetration capacity (functional homologue). A portion of the CDR region can consist of a CDR region which is free of one or more terminal amino acids, in particular one, two or three terminal amino acids. It may also be a CDR region where one or more internal residues have been deleted or substituted by other amino acids, preferably amino acids of the same nature (for example basic amino acids). Advantageously, less than 30% of the internal residues of

the CDR region are modified, preferably less than 20% and more preferably less than 15%.

Preferred polypeptides of the invention are thus polypeptides comprising all or a portion of a CDR3 region of an antibody. By way of illustration, CDR3 regions with sequence SEQ ID N° 1, 2, 3, 8 or the sequences shown in Figure 2 and Figure 3 or any functional homologue can be cited.

The antibody fragments can themselves constitute the polypeptide of the invention. They can also be modified by adding residues to one or both of their extremities. In particular, it may be advantageous to add amino acids which give the fragment, in particular the CDR region, a better spatial configuration. It may also be advantageous to add one or more essentially basic amino acids, lysine and/or arginine in type, to stabilise the polypeptide and increase its interaction with the cell membranes. Further, as indicated above, the polypeptides of the invention may comprise several regions of an antibody chain, such as a CDR2 region and a CDR3 region. These regions can in particular be fused together or spaced by amino acids as described above.

Particular polypeptides of the invention are polypeptides comprising a CDR3 region of an antibody or polypeptides essentially comprising a fusion between the CDR3 region of an antibody and the CDR2 region of an antibody. Examples of such polypeptides are the CDR3 polypeptides and the CDR2-3 polypeptide the sequences for which are given in the Examples. One preferred CDR2 region according to the invention is represented by the following sequence (m=0 or 1), or any sequence having homology higher than 50%, preferably 65% with such sequence.

$(Val)_m$-$(Ala)_m$-Tyr-Ile-Ser-Arg-Gly-Gly-Val-Ser-Thr-Tyr-Tyr-Ser-Asp-Thr-Val-Lys-Gly-$(Arg)_m$-$(Phe)_m$ = SEQ ID N°4.

Experiments carried out with these polypeptides, in particular polypeptides comprising the CDR3 region, and more particularly those comprising the CDR3 region and the CDR2 region, clearly show that:

1) incubating PtK2, HeLa or 3T3 cells for one hour with complete culture medium (10% foetal calf serum) containing the polypeptide is sufficient for the polypeptide to be massively transported into the cytoplasm of all the cells and into the nucleus of a large proportion of these cells.

2) When cells are incubated for 2 hours in complete culture medium containing pre-formed peptide-streptavidin complexes coupled to peroxidase (MW ≥ 100000) or peptide-streptavidin coupled to alkaline phosphatase (MW ≥ 180000), the corresponding enzymes are detected in the cytoplasm of all of the cells of the culture and weakly to intensely detected in the majority of the nuclei of these cells. No intracellular coloration is observed when the cells are incubated in the presence of streptavidin coupled with peroxidase, streptavidin coupled with alkaline phosphatase or with streptavidin or the enzymes in their native forms.

The polypeptides of the invention, in particular of type CDR3 and CDR2-3, and their peptide-streptavidin-enzyme complexes are transported in large quantities into a large proportion of human peripheral cells and particularly into activated T lymphocytes.

In general, the polypeptides of the invention can be constructed using different techniques which are known to

the skilled person (supra), starting from any given antibody, in particular any given monoclonal antibody.

Preferably, the polypeptides of the invention are obtained by chemical synthesis or are constructed from a fragment or several fragments of one or more penetrating antibody(ies), preferably a penetrating monoclonal antibody. The existence of antibodies which can penetrate inside cells and in particular into the nuclei of human lymphocytes when these cells are incubated *in vitro* in a culture medium containing a serum originating from patients with disseminated lupus erythematosus (DLE) was reported for the first time by Alarcon-Segovia et al. In 1978 (Nature, 271). Recently, this type of antibody has been detected in the lupus mouse MRL lpr/lpr, but also in the NZB mouse with an autoimmune hemolytic disease syndrome and even in the normal BALB/c mouse. Certain monoclonal antibodies prepared from the spleen of these mice have been shown to be capable of penetrating *in vitro* into the nucleus of cells maintained in culture (Vlahakos et al., J. Am. Soc. Nephrol. 2 (1992) 1345; Eyal Raz et al., Eur. J. Immuno. 23 (1993) 383). Further, it has been shown that these antibodies are also capable, when injected into mice, of penetrating into several types of cells, and are found in their nuclei (Okudaira et al., Arthritis and Rheumatism, 30 (1987) 669).

In general, any antibody can be selected with a view of determining its penetrating character. This selection can be made, for example, using a cell penetration test comprising initial incubation of the antibody under study in the presence of cells, in particular cells into which it is desired to transport a substance of interest, followed by fixing and permeabilisation of these cells,

revealing the presence or otherwise of this antibody in the plasmic membrane, the cytoplasm or in the immediate proximity of the nucleus or even in the nucleus. Revealing can, for example, be effected by incubation with a second labelled antibody, directed against the test antibody, followed by detection of the immunological reaction of the antigen-antibody type between these two antibodies. Such a test has been described in detail, for example in French patent FR-9508316.

Still more preferably, the fragment of antibody used to construct a polypeptide of the invention is a fragment of a polyreactive antibody, in particular penetrating and polyreactive. A polyreactive antibody is an antibody which is capable of recognising several different antigens. In general, such antibodies have a particularly high affinity for a particular type of antigen and are capable of recognising one or more other antigens with a lower affinity. The polyreactivity of antibodies can be demonstrated by any conventional immunological technique, such as the methodology described by Sibille et al. (Eur. J. Immuno. 1997, 27: 1221-1228).

Advantageously, the polyreactive antibodies used in constructing the polypeptides of the invention are capable of reacting with nucleic acids, free or complexed with proteins (anti-DNA antibodies). More preferably, these are antibodies which are capable of reacting with nucleic acids and which recognize, among others, proteins such as Tat or Rev of the HIV retrovirus and/or surface markers such as CD3, CD4, CD8, CD19 or CD34.

To construct a polypeptide of the invention, the selected antibody is then used as follows:

a) if the sequence of the variable region of the heavy or light chain of this antibody is not accessible, it is determined in a first stage. Conventional sequencing techniques can be used, as illustrated in the examples;

b) the CDR regions are localised by sequence alignment with other sequences of antibody chains;

c) fragments of this sequence are prepared, or the polypeptides corresponding to regions of this sequence are synthesised. To this end, any conventional technique which is known to the skilled person can be used (peptide sequencers, using a restriction enzyme, ligases, etc…);

d) the fragment obtained may be modified by addition, deletion or substitution of amino acids;

e) the cell penetration capacity of the polypeptide obtained is then tested under the conditions described above.

Optionally, steps c), d) and e) or d) and e) are repeated so as to improve the penetration efficacy or the general properties of the polypeptides of the invention.

In a supplemental subsequent step f), the polypeptide obtained, with the capacity to penetrate into cells, is then used in a coupling reaction with a given substance to generate a vector as will be defined below.

Preferably in step c), the prepared fragments comprise all or a portion of the CDR3 region of an antibody.

In step d), modifications can, for example, consist of introducing certain supplemental amino acids, either simply for technical reasons (ease of synthesis, coupling

between different regions, etc) or for structural or physicochemical reasons. Concerning amino acids for "filling", amino acids which are relatively neutral on the structural and physicochemical level are advantageously used. Regarding structural reasons, as indicated above, adding residues can improve the conformation of the polypeptide and thus potentialise its activity. Further, it may also be desirable to increase the basic nature of the polypeptides.

To this end, to improve the compaction properties of the polypeptides of the invention, in particular as regards nucleic acids, the active site of the antibody being kept free, polypeptides have been constructed comprising a fragment of an antibody and lysine residues. More specifically, polypeptides have been constructed comprising a fragment of an antibody having all or part of a CDR3, and a polylysine. Advantageously, the number of lysine residues is less than 30, more preferably between 10 and 20.

The results presented in the examples confirm the penetration properties of these polypeptides, and their capacity for effective transport of substances of interest, in particular nucleic acids.

Whatever the additions made, the polypeptide of the invention as prepared, for example, using the above protocol, preferably comprises at most 100 amino acids. Still more preferably, it comprises 3 to 60 amino acids, preferably 3 to 40 amino acids.

In a further aspect, the invention concerns the use of a polypeptide as defined above to transfer substances into cells, *in vitro*, *ex vivo* or *in vivo*.

In a still further aspect, the invention concerns a vector for transferring a substance into a cell,

characterized in that it comprises a polypeptide as defined above to which said substance is coupled.

The coupled substance can be any product of interest, in particular a pharmaceutical or agro-alimentary product. In particular, it may be a nucleic acid, such as a ribonucleic acid or a deoxyribonucleic acid. This nucleic acid can also be from a variety of origins, in particular human, viral, animal, eukaryotic or prokaryotic, plant, synthetic, etc... This nucleic acid can also be a variety of sizes, from a simple oligonucleotide to a genome or a fraction thereof. In particular, it may be a viral genome or a plasmid. The substance can also be a protein, such as an enzyme, hormone, cytokine, apolipoprotein, growth factor, etc.. A particular type of substance is represented by antigens. As indicated below, the polypeptides of the invention can advantageously act as an adjuvant and stimulate the immune response directed against an antigen.

More generally, the substance can be any active principle of a drug, be it a chemical, biochemical or synthetic product.

To enable its transfer into a cell, said substance is thus coupled to a polypeptide of the invention.

The term "coupled" as used in the invention means any type of interaction enabling a physical association between the substance and the polypeptide. Preferably, however, the interaction is sufficiently stable for the vector not to dissociate before cell penetration. For this reason, the preferred coupling is covalent coupling.

Covalent coupling can be effected by different techniques which are known to the skilled person. In particular, it can be effected using maleimide,

succinimide, peptide, disulphide or thioether bonds. Reference should be made in this respect to "Bioconjugate Techniques" by Greg T. HERMANSON (Academic Press, 1996).

A particular method consists, for example, of adding a cystein residue which can be readily used for disulphide, thioether, amine or acid bonds to one extremity of the polypeptide of the invention. A further approach consists of chemically coupling a biotin group, which then enables any substance bonded to streptavidin to be coupled. Coupling can also be effected using p-benzoquinone (FR-7537392 and US-A-4 925 921, for example).

In general, any chemical, biochemical, enzymatic or genetic coupling method which is known in the literature can be used.

Further, a vector of the invention can comprise a polypeptide as described above to which a number of identical or different substances are coupled.

The examples below clearly demonstrate that the polypeptides of the invention have the ability not only to penetrate into cells, but also to transport substances of interest thereto. The examples demonstrate enzyme type protein transport. It should be understood that enzymes can be substituted by any other molecule of interest such as nucleic acids, peptides or drugs, under the same conditions.

Further, the fact that the polypeptides of the invention enable massive transport of proteins into cells has also prompted the Applicant to examine the possibility of using them as an intracellular antigen transport agent, endowing them with an adjuvant effect and leading to an increase in the immune response against these antigens. Thus mice received several injections

with the streptavidin-peroxidase conjugate alone or complexed with a polypeptide of the invention. The results obtained show that the use of a polypeptide of the invention can increase by on average 4 to 8 times the titer of anti-streptavidin antibodies and anti-peroxidase antibodies.

In a still further aspect, the invention provides a cell, in particular a eukaryotic cell, containing a polypeptide or a vector as defined above. This cell is advantageously a mammalian cell, in particular an animal or human cell. In particular, it may be a cell of the hematopoietic system, such as a progenitor cell or a strain cell or a lymphocyte cell (T, B). It may also be a cell presenting the antigen such as a macrophage or dendritic cell.

To this regard, the invention also concerns a method for transferring a substance into a cell *in vitro*, *ex vivo* or *in vivo* comprising:

- coupling said substance to a polypeptide as defined above; and
- bringing the cell into contact with the product of said coupling.

For *in vitro* or *ex vivo* use, contact can be effected by simple incubation of the cells with the coupling product (vector). For *in vivo* use, contact is generally effected by administering the coupling product (vector) to the organism under consideration.

To this end, the invention also concerns a pharmaceutical composition comprising, in association with a physiologically acceptable vehicle, a vector as defined above in which the substance is an active principle of a drug.

The invention still further concerns a vaccine comprising, in association with a physiologically acceptable vehicle, a vector as defined above in which the substance is an antigen.

The present invention will now be described in more detail using the following non limiting examples which are provided by way of illustration.

Key to Figures

Figure 1: Diagram of the structure of an antibody.

Figure 2: Peptide sequence of the variable regions of the heavy chain of penetrating monoclonal antibody. The CDR regions are shown. "-" means identical amino acids.

Figure 3: Sequence for CDR2 and CDR3 regions of the VH domains in cell penetrating anti-DNA antibody.

Figure 4: ELISA demonstration of the *in vivo* adjuvant effect of the polypeptides of the invention.

Figure 5: DNA transfection activity of the CDR2-3-PL19 peptide in CCL39 cells.

Method and apparatus

Mice and cell lines:

BALB/c (NZB x NZW) F1 mice were kept in the animal house at the Institut Pasteur. Cells from different species and from different tissues were used: PtK2 cells (kidney fibroblasts), GMA-32 cells (hamster lung), 3T3 cells (mouse embryo fibroblasts), CCL39 cells (hamster fibroblasts), HeLa cells (human cervical carcinoma), VERO cells (monkey kidney), HEp-2 cells (human larynx carcinoma), JURKAT cells and CEM cells (human T lymphoblasts) all available from the ATCC Collection. These different cell types were cultivated in RPMI medium

or in DMEM medium containing 10% of inactivated foetal calf serum and supplemented with L-glutamine, sodium pyruvate and non-essential amino acids and antibiotics (complete culture medium) at 37°C in a moist atmosphere containing 5% CO_2.

Monoclonal antibodies

The preparation and isolation of monoclonal antibodies J20.8, F4.1 and F14.6 have been described in French patent application FR-9508316.

These antibodies were purified on a protein A sepharose column (Ey et al., Immunochemistry 15 (1978) 429). The polyreactivity of these purified antibodies as regards double stranded DNA and other antigens was tested using ELISA employing the methodologies described in the literature (Guilbert et al., J. Immunol. 128 (1982) 2779).

Examples

1. Sequencing of monoclonal antibodies

The nucleotide sequence of the VH and VL regions of monoclonal antibodies J20.8, F4.1 and F14.6 were determined. To this end, total RNA was extracted from hybridoma cells using the guanidine thiocyanate technique (Schwartz et al., Biol. Cell. 73 (1991) 7) then separated by formaldehyde/agarose gel electrophoresis. The messenger RNA obtained was then transformed into complementary DNA using a reverse transcriptase kit (Life Technologies, Eragny, France) and used as a primer in amplification reactions (PCR) using Taq DNA polymerase (Boehringer, Mannheim, Germany) following the manufacturer's instructions. The oligonucleotide primers used to generate the complementary DNA were:

- firstly, a primer corresponding to the conserved sequences of IgG2a immunoglobulins:

  5'-GTTCTGACTAGTGGGCACTCTGGGCT (SEQ ID N° 11)

- and secondly, four primers for the VH region:

  5'-GAGGTTCAGCTCGAGCAGTCTGGGGC (SEQ ID N° 12)

  5'-GAGGTGAAGCTCGAGGAATCTGGAGG (SEQ ID N° 13)

  5'-GAAGTGCAGCTCGAGGAGTCTGGGG (SEQ ID N° 14)

  5'-GAGGTTCAGCTCGAGCAGTCTGGAGC (SEQ ID N° 15)

The PCR amplification products were then purified using a Geneclean kit (Bio 101, Vista, CA). Chemical sequencing was carried out by Genome Express (Grenoble, France). The nucleotide sequences were analysed using GENBANK and EMBL databases held at the Institut Pasteur (Information Science Unit) using GCG sequence analysis software (GCG) (Devereux J., "The GCG Sequence Analysis Software Package", 1989), and the corresponding amino acid sequences were deduced.

The sequence for the VH regions of these antibodies is shown in Figure 2. Alignment of these sequences enabled the CDR regions (CDR1, CDR2 and CDR3) present in these sequences to be localised. This alignment also demonstrated the existence of a substantial structural homology between the CDR2 regions and the common structural characteristics between the CDR3 regions, in particular the presence of basic residues (arginine and lysine). The sequences for the CDR2 and CDR3 regions of other antibodies are shown in Figure 3.

2. Construction of penetrating polypeptides

Starting from the sequences shown in Figure 1, different polypeptides comprising all or a portion of the CDR3 region and/or the CDR2 region of the antibodies were prepared. Synthesis was carried out by peptide synthesisers (see Method and Apparatus section). The

following polypeptides were synthesised, where m is 1 or 0:

CDR3:

SEQ ID n° 1:

$(Thr)_m$-$(Arg)_m$-$(Gln)_m$-Lys-Tyr-Asn-Lys-Arg-Ala-$(M\text{-}D\text{-}Y\text{-}W\text{-}G\text{-}Q\text{-}G\text{-}T)_m$

A variation of this sequence is, for example the sequence $TRQKYNKRA(MDYWGQGT)_m$. A further variation (functional homologue) is, for example, the sequence Ala-Arg-Gln-Lys-Tyr-Asn-Lys-Arg-Ala-Met-Asp-Tyr (SEQ ID n° 8).

SEQ ID n° 2:

$(Thr)_m$-$(Arg)_m$-$(Gln)_m$-Lys-Tyr-Gly-Lys-Arg-Gly-$(M\text{-}D\text{-}Y\text{-}W\text{-}G\text{-}Q\text{-}G\text{-}T)_m$

A variation of this sequence is, for example the sequence $TRQKYNKKRG(MDYWGQGT)_m$.

SEQ ID n° 3:

$(Thr)_m$-$(Arg)_m$-$(Gln)_m$-Ala-Arg-Ala-Thr-Trp-Asp-Trp$(F\text{-}A\text{-}Y\text{-}W\text{-}G\text{-}Q\text{-}G\text{-}T)_m$

A variation of this sequence is, for example the sequence $TRGARATWDW(FAYWGQGT)_m$.

In sequences 1 to 3 above, MDYWGQGT = Met-Asp-Tyr-Trp-Gly-Gln-Gly-Thr and FAYWGQGT = Phe-Ala-Tyr-Trp-Gly-Gln-Gly-Thr. Further, the formula $(a\text{-}b\text{-}c)_m$ means that a single, some or all of the residues mentioned in brackets are present or are not present.

CDR2:

SEQ ID n° 4:

$(Val)_m$-$(Ala)_m$-Tyr-Ile-Ser-Arg-Gly-Gly-Val-Ser-Thr-Tyr-Tyr-Ser-Asp-Thr-Val-Lys-Gly-$(Arg)_m$-$(Phe)_m$-$(Thr)_m$ (CDR2(1)). A variation of this sequence is, for example, the sequence VAYISRGGVSTYYSDTVKGRF or VAYISRGGVSTYYSDTVKGRFT.

SEQ ID n° 5:

$(Val)_m$-$(Ala)_m$-Tyr-Ile-Ser-Arg-Gly-Gly-Gly-Ile-Phe-Tyr-Tyr-Glu-Asp-Ser-Ile-Lys-Gly-$(Arg)_m$-$(Phe)_m$ (CDR2(2)). A variation of this sequence is, for example, the sequence VAYISRGGIFYYQDSIKGRF.

SEQ ID n° 6:
$(Val)_m$-$(Ala)_m$-Ala-Ile-Ser-Arg-Gly-Gly-Gly-Tyr-Ser-Tyr-Tyr-Leu-Asp-Ser-Val-Lys-Gly-$(Arg)_m$-$(Phe)_m$-$(Thr)_m$-$(Ile)_m$ (CDR2(3)). A variation of this sequence is, for example, the sequence VAAISRGGGYSYYLDSVKGRFTI.

SEQ ID n° 7 (p3 or Pf4.1 peptide):
Val-Ala-Tyr-Ile-Ser-Arg-Gly-Gly-Val-Ser-Thr-Tyr-Tyr-Ser-Asp-Thr-Val-Lys-Gly-Arg-Phe-Thr-Arg-Gln-Lys-Tyr-Asn-Lys-Arg-Ala. A variation of this sequence is, for example, the sequence VAYISRGGVSTYYSDTVKGRFTRQKYNKRAVAY.

<u>Functionalised CDR2-3</u>:
Biotinyl-Val-Ala-Tyr-Ile-Ser-Arg-Gly-Gly-Val-Ser-Thr-Tyr-Tyr-Ser-Asp-Thr-Val-Lys-Gly-Arg-Phe-Thr-Arg-Gln-Lys-Tyr-Asn-Lys-Arg-Ala-
(corresponding to the sequence SEQ ID n° 7).

Cys-Val-Ala-Tyr-Ile-Ser-Arg-Gly-Gly-Val-Ser-Thr-Tyr-Tyr-Ser-Asp-Thr-Val-Lys-Gly-Arg-Phe-Thr-Arg-Gln-Lys-Tyr-Asn-Lys-Arg-Ala- (SEQ ID n° 9).

An active group (SH) was introduced into sequence SEQ ID n° 9 via cystein to enabling coupling to another substance.

<u>Functionalised CDR2</u>:

Biotinyl-VAYISRGGVSTYYSDTVKGRFT (Biotinyl-Val-Ala-Tyr-Ile-Ser-Arg-Gly-Gly-Val-Ser-Thr-Tyr-tTyr-Ser-Asp-Thr-Val-Lys-Gly-Arg-Phe-Thr), corresponding to sequence SEQ ID n°4.

Functionalised CDR3:
Biotinyl-Ala-Arg-Gln-Lys-Tyr-Asn-Lys-Arg-Ala-Met-Asp-Tyr (corresponding to sequence SEQ ID n° 8).

3. Study of the penetration of polypeptides into cells

Cultured PtK2 fibroblasts seeded the day before in an amount of 5 x 10^4 cells per well onto glass sheets, were incubated at 37°C, 1-18 hours in complete RPMI 1640 culture medium (or DMEM) (10% foetal calf serum, 2 mM L-glutamine and 1 mM of sodium pyruvate) containing a biotinylated polypeptide of the invention (5-20 µg/ml). The cells were then washed with PBS and fixed with 2% of p-formaldehyde at 4°C for 10 minutes then washed with PBS.

The cells were then incubated with a solution of streptavidin conjugated with 5 µg/ml of peroxidase in PBS for 30 minutes, then washed with PBS and incubated in the peroxidase cytochemical substrate (diaminobenzidene + H_2O_2). After washing, the cells were examined microscopically.

The results obtained show that after 1 hour of culture, the polypeptides comprising all or a portion of a CDR3 were visible by peroxidase coloration in the cytoplasm of all of the cells and in the nucleus of a large number of cells. The results also show that the polypeptide CDR2-3 (in particular the pF4.1 polypeptide with sequence SEQ ID n° 7) penetrated massively and rapidly into the cells and most reached the nucleus of

said cells. These results thus show that it is possible to generate polypeptides with a high cell penetration capacity from a CDR3 type fragment.

4. Cell penetration of polypeptide-streptavidin vectors coupled to enzymes

This example illustrates how the polypeptides of the invention can be coupled to an active substance and used to transport said substance into cells.

The vectors were prepared by incubating 1.4 µg of biotinylated CDR2-3 polypeptide (pF4.1) with 10 µg of streptavidin conjugated with peroxidase or with alkaline phosphatase in a volume of 10 µl for 15 minutes at laboratory temperature. The mixture was then diluted in 0.5 ml of complete culture medium before being deposited on the cells in culture. After 2 hours of culture, the cells were washed with PBS, fixed with p-formaldehyde, washed then incubated in the peroxidase cytochemical substrate (diaminobenzidine + H_2CO_2) or that of alkaline phosphatase (Naphthol AsMx + Fast Scarlet tetrazolium salt).

The results obtained show that the corresponding enzymes were detected in the cytoplasm of all of the cultured cells and weakly to intensely in the majority of the cell nuclei. In contrast, no intracellular coloration was observed when the cells were incubated in the presence of streptavidin coupled with peroxidase, streptavidin coupled with alkaline phosphatase or with streptavidin or the enzymes in their native forms.

5. Construction and activity of a polypeptide comprising supplemental lysine residues

A CDR2-3-PL19 (polylysine) was synthesised and purified (ALTERGEN). The sequence of the polypeptide is as follows:
(NH2-(K19)-VAYISRGGVSTYYSDTVKGRFTRQKYNKRA-COOH)
SEQ ID n° 10.

CCL39 cells (hamster fibroblasts) (5 x 10^4 cells) were placed in 24 well culture plates for 18 hours before transfection in MEM + 10% FCS (foetal calf serum) culture medium. Transfections were carried out in MEM + 10% FCS with no other auxiliary agent (CHLOROQUINE). The peptide-PL and free polylysine PL (corresponding to 19 lysines) were complexed with the pCMVLUC plasmid (respectively 24 µg and 70 µg per 6 µg of plasmid) for 30 minutes. The complex was then added dropwise to the CCL39. After incubating for 5 hours, the medium was replaced with fresh medium. Luciferase expression was assayed 24 hours later. The cells were washed twice with PBS. After washing, the cells were lysed with 100 µl of lyse buffer (PROMEGA) for 10-15 minutes. The cells were then centrifuged for 7 minutes at 4°C to remove cellular debris. 20 µl of this lysate was mixed with 100 µl of luciferase buffer (PROMEGA). The relative luciferase units (RLU) were recorded on a LUMAT LB9501 (BERTHOLD). The protein concentration was determined using a BIORAD PROTEIN ASSAY-1 kit and the amount of luciferase in each sample was normalised per mg of protein, each transfection being carried out three times.

The results obtained are shown in Figure 5. They show that in complete medium and with no auxiliary agent, the peptide-PL transfects with an efficacy of 2 x 10^6 RLU/mg of proteins, i.e., about 1000 times more than polylysine alone and more than a peptide recently

described (Wadhwa et al., Bioconjugate Chem. 1997, 8:81-88), but where the activity was dependent on the presence of 100 μM of chloroquine.

Transfecting cells with the CDRK19-P3 polypeptide is thus particularly advantageous since it can be carried out in a complete culture medium and in the absence of auxiliary agent. Current transfection systems using polylysine all require the addition of an auxiliary agent, usually chloroquine which is toxic for the cells. This chloroquine prevents degradation in the lysosomes of conjugate-polylysine complexes internalised by the conventional endocytosis route.

In contrast, the present invention does not require the use of such auxiliary agents.

6. Use of a polypeptide as an immunoadjuvant

The vector was formed by incubating 14 μg of biotinylated CDR2-3 polypeptide + 40 μg of streptavidin conjugated with peroxidase (Sigma) for 15 minutes then diluting in 0.1 ml of PBS before being injected into each mouse. Injection was via the pads. The control mice received 40 μg of streptavidin conjugated with peroxidase in 0.1 ml of PBS.

The mice were bled every week. A repeat injection was carried out under the same conditions one month after the first injection. An ELISA test showed that the mice which had received the CDR2-3-streptavidin conjugated with peroxidase complex responded with anti-streptavidin and anti-peroxidase IgG antibodies, but with very few IgM, with substantially higher values than those which had received streptavidin conjugated with peroxidase alone and from the 14^{th} day (Figure 4). The repeat injection caused an increase in the antibody titer in the two groups, but the values were always higher in the

group which had received the complex. On the basis of these results, it thus appears that under the test conditions, the polypeptides of the invention are capable of increasing the titer of antibodies directed against a given antigen by a factor of at least 4 to 8.

The same experiments can be reproduced using not a protein as the antigen but a nucleic acid coding for said antigen. Further, these experiments can also be repeated under the same conditions with a polypeptide comprising supplemental basic residues, in particular a polylysine.

This set of results clearly demonstrates that the polypeptides of the invention, comprising an antibody region, preferably comprising all or a portion of a CDR3, are capable (i) of effectively penetrating into cells; (ii) of transporting substances thereto, in particular large size substances; (iii), of acting as an adjuvant *in vivo* by stimulating the immune response against a given antigen; and (iv) of exerting an antiviral activity. Further, the polypeptides of the invention even appear to be able to transport substances to the cell nuclei, which is of obvious interest when the substances are nucleic acids or molecules acting on nucleic acids. Further, the polypeptides of the invention appear to use a cell penetration mechanism which is different from the majority of vectors used up to the present time. In particular, the polypeptides of the invention appear to escape the lysosomes, which constitutes an additional advantage in that in general, substantial degradation occurs in those cellular compartments.